ゾルゲル法による生物学活性ガラス
Biologically Active Sol-Gel Derived Glasses

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概　要

ゾルゲル法は無機マトリックス多孔体中に分子を固定化することのできる液相合成法である。ゾルゲルマトリックス中に酵素やタンパク質の生体分子をうまくカプセル化できることが実証された。固定化されたタンパク質は生物学活性を維持し，ある場合には酵素はカプセル化により安定性を増した。さらにゾルゲル法が柔軟性を有するので，これらの物質をデバイス構造に組み込むことが出来る。ここではゾルゲルによりカプセル化した生体分子の特性とバイオセンサーでの用途について記述する。この研究により生物学活性のゾルゲル材料はセンサー素子にとって魅力ある材料であることが示された。

Abstract

Sol-gel chemistry is a liquid phase synthesis method for preparing inorganic oxides whereby molecules can be immobilized in the pores of an inorganic matrix. The successful encapsulation of biomolecules such as enzymes and other proteins within sol-gel matrices has been well documented. The sol-gel immobilized proteins retain biological activity, and in some cases, enzymes experience increased stability upon encapsulation. In addition, the processing flexibility of sol-gel chemistry enables these materials to be integrated into device structures. This article reviews the properties of sol-gel encapsulated biomolecules and describes their use in biosensing applications. It is apparent from this work that bioactive sol-gel materials are an attractive material platform for sensing elements.

1. Introduction

The sol-gel process is a chemical synthesis technique for preparing amorphous inorganic solids. The synthesis generally involves the use of liquid metal alkoxides, $M(OR)_n$ (where M is Al, Si, Ti, etc. and R is an organic group), which undergo hydrolysis and condensation polymerization reactions to give solid gels. The solution nature of the process enables the materials to be prepared as films, fibers or as bulk materials. Beginning with metal alkoxide(s) as a precursor and in the presence of water and a catalyst, hydrolysis and condensation reactions occur simultaneously to form a colloidal sol with alcohol as a by-product. An example of the formation of a silica sol-gel material is as follows:

$$Si(OC_2H_5)_4 + 4H_2O \longrightarrow Si(OH)_4 + 4C_2H_5OH \quad (1)$$

$$n\,Si(OH)_4 \longrightarrow nSiO_2 + 2nH_2O \quad (2)$$

There are a variety of factors which affect the hydrolysis and condensation reactions and final

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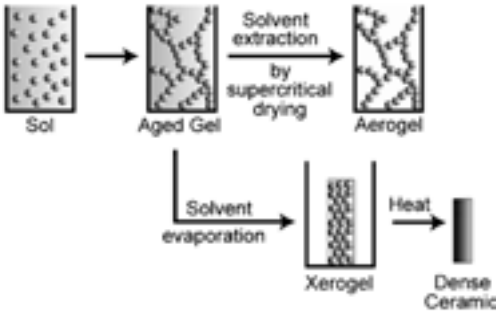

Fig. 1 Overview of sol-gel processing in the fabrication of wet (aged) gels, aerogels, and xerogels. (Adapted from reference 1).

microstructure of the gel, including pH, temperature, nature of alkoxide, amount of alcohol, ratio of alkoxide to water to alcohol, and type of catalyst used. After the sol-to-gel transition, there is a sharp increase in viscosity and the polymer structure becomes rigid. The sol-to-gel transition is irreversible, with essentially no change in volume. The material after gelation is composed of two distinct phases, amorphous silica particles (5–10 nm in diameter) and an interstitial liquid phase. The method of drying (solvent evaporation or extraction) can greatly affect the final matrix structure, pore size, and pore volume, as shown in Figure 1.[1] The resulting material can be in the form of a monolith, thin film, or fiber, depending on the fabrication method. For bioactive glasses, retaining the interstitial pore liquid is necessary as biomolecules need an aqueous environment to maintain biological function. Therefore, bioactive glasses generally exist in the ''wet'' or ''aged'' gel state, wherein pores are filled with liquid buffer.

There are a variety of methods for immobilizing biomolecules including entrapment, microencapsulation, covalent attachment and adsorption. The present article describes the immobilization of biomolecules using the sol-gel process. In this method, biomolecules are encapsulated in the pores of an inorganic material that is prepared using sol-gel chemistry. The process can be carried out at room temperature under biologically compatible conditions. By adding dopant biomolecules to a hydrolyzed liquid sol, the final solid gel contains biomolecules trapped in a solvent (buffer) rich environment in the pores of the mesoporous network.

The encapsulation of proteins and other biomolecules in sol-gel derived glasses has stimulated much interest in the engineering community because delicate biomolecules can be immobilized in a solid-state material that is rugged and mechanically, thermally and chemically stable. Moreover, silica-based matrices have excellent optical transparency, making it possible to use these materials as optical sensors. Due to the nature of the sol-gel process, the amorphous solid network forms around the high molecular weight biomolecules, confining them within the pores of the solid-state matrix. Although the relatively large biomolecules (on the order of tens to hundreds of angstroms) are immobilized within the inorganic network, small ions and molecules are able to diffuse into and out of the network. Therefore, the sol-gel encapsulated biomolecules retain their native biological activity and spectroscopic properties, and they can respond to chemical changes in their environment (i.e. they can be used as sensors). A diverse range of proteins has been immobilized in sol-gel derived materials including globular and membrane-bound proteins, enzymes and other biosystems. Research results on bioactive glasses prepared by the sol-gel method have been described and summarized in a number of published reviews.[2~5]

2. Stabilization of Biomolecules by Sol-Gel Encapsulation

One of the most important benefits of sol-gel immobilization that has emerged is the ability to stabilize biomolecules through encapsulation. Accumulated results give indirect evidence that a biomolecule designs a self-specific pore as the silicate network forms around it during hydrolysis and condensation reactions in sol-gel synthesis.[6] There is a silicate ''cage'' that defines the pore according to the size and shape requirements of the biomolecule. Consequently, the biomolecule prevents its surrounding pore from collapsing, and the matrix prevents the biomolecules from unfolding and aggregating, two common denaturation pathways. In addition, the inorganic matrix effectively prevents the encapsulated biomolecule from contact with proteases or microorganisms. In the following paragraphs, we briefly describe the enhanced thermal, storage and chemical stabilities reported for biomolecules as a result of sol-gel immobilization.

A marked improvement in thermal stability as a result of sol-gel encapsulation has been observed for three flavoprotein oxidases, glucose oxidase (GOx), lactate oxidase (LOx), and glycolate oxidase (GLyOx).[7] The extent of the stabilization was impressive, as the half-life of glucose oxidase (GOx) at 63℃ was increased 200-fold upon sol-gel encapsulation as compared to enzyme in water. In contrast, LOx and GLyOx were initially destabilized by sol-gel encapsulation, due to electrostatic interactions between the enzymes with the highly charged surfaces of the sol-gel matrix, which proved detrimental to enzyme activity. If the enzymes were electrostatically complexed *prior* to sol-gel immobilization, however, enhanced thermal stability was once again observed. LOx experienced a 150-fold increase and GLyOx experienced a 100-fold increase in enzyme half-life at 63℃ as compared to enzyme in water.

Increased thermal stability has also been observed in the heme protein cytochrome-c (cyt-c). Thermally induced unfolding of proteins in solution, in general, exhibits a sharp transition over a small temperature range, and the transition point at which half of the molecules are denatured is termed T_m. T_m for cyt-c in liquid buffer was～65℃, whereas T_m for cyt-c in the sol-gel matrix was at least 90℃.[6]

A third representative protein where enhanced thermal stability has been observed is the enzyme creatine kinase (CK).[8] We carried out a study which compared the activity of CK in sol-gel silica gels and in liquid buffer at 37℃, 47℃, and 60℃. In all cases the enzyme activity of the sol-gel encapsulated CK was retained for a much longer time than in solution. For example, at 60℃, CK in buffered solution had no activity after one hour, whereas the sol-gel immobilized CK retained 50% of its activity after 5 hours. Measurement of the thermal transition in CK confirmed the enhanced stability, as T_m was ～75℃ for CK in liquid buffer, whereas T_m of the sol-gel encapsulated enzyme was ＞90℃.

In view of the thermal stability results, it is not surprising that sol-gel encapsulation is beneficial for extended storage of enzymes and other proteins at or below room temperature. In experiments with cholinesterase, sol-gel immobilized enzyme retained enzymatic activity during storage significantly better than the enzyme in buffer solution. The sol-gel encapsulated enzyme retained essentially full activity after 40 days storage whereas the enzyme in liquid buffer experienced a steady decline from the very beginning, and after 30 days, the en-

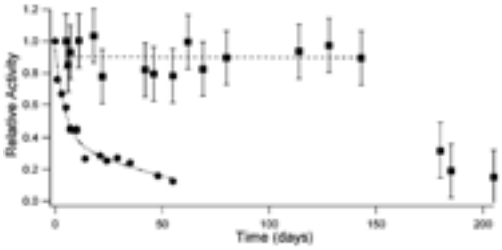

Fig. 2 Relative activity as a function of time for creatine kinase when stored at room temperature in pH 7 HEPES buffer solution (●) and in sol-gel silica (■) shows significantly improved storage stability as a result of sol-gel encapsulation.

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zyme in liquid buffer had essentially no activity. A similar effect was observed in the enzyme CK. At room temperature, the activity of encapsulated enzyme decreased to 50% of its initial value after more than five months, whereas in buffer solution it decreased to 50% after ten days, as shown in Figure 2.[8)]

Improved chemical stability has also been demonstrated. Antibodies immobilized via the sol-gel route retained their ability to bind antigen after acid exposure to a greater extent as compared to antibodies immobilized via traditional surface attachment. After exposure to 0.01N HCl (pH ～2) for 24 hours, sol-gel encapsulated anti-trinitrotoluene (anti-TNT) antibodies experienced essentially no loss in their ability to bind TNT whereas antibodies immobilized via traditional surface attachment experienced >30% loss.[9)]

Sol-gel encapsulation also improved the chemical stability of cyt-c when immersed in alcohol solutions.[10)] Optical absorption studies indicated that this heme protein partially denatured (unfolded) in buffered solutions with less than 60 vol% methanol (MeOH), with the degree of denaturation increasing as the amount of methanol increased. Furthermore, at concentrations >60 vol% MeOH, aggregation occurred, depending on the buffer. When encapsulated in silicate gels, however, protein denaturation (unfolding) due to MeOH was fully reversible. That is, there was some unfolding on exposure to MeOH, however, the protein reverted to its native form when samples were then soaked in pure buffer. Moreover, protein aggregation did *not* occur for the sol-gel immobilized protein even if the gels were soaked in pure methanol for several weeks. These results confirm that isolating biomolecules in the pores of the matrix prevented aggregation by limiting the long-range mobility of the protein. Local motions within the pore environment are not affected and the protein retains its characteristic reactivities.

The collective results have shown that sol-gel encapsulation has led to a marked improvement in stability for a number of enzymes and other proteins. The exact mechanism is not yet understood, and there are important molecule-matrix interactions that must be considered for the sol-gel process. The first is the electrostatic interactions between the biomolecule, especially its ''active site'', and the silicate matrix, as both are usually highly charged.[7)] Another consideration is that the observed rates of reactions are likely to be lower due to diffusion limitations. The target analyte must diffuse through the pores of the silicate matrix, leading to a slower response time. Finally, depending on the size of the immobilized biomolecule, the effect of protein crowding may result in destabilization of the protein conformation.[11)] Despite these considerations, the evidence to date indicates that sol-gel immobilization is a viable

means of ''ruggedizing'' biomolecules. Once encapsulated in a solid glass, the biomolecules can be handled more easily and exposed to harsher environments without severe degradation of their properties.

3. Biosensing Using Biologically Active Sol-Gel Glasses

The potential use of bioactive sol-gel based materials as sensing elements has made these materials the subject of intensive study. In our earlier work, we demonstrated that antibodies can be encapsulated in optically transparent sol-gel silica glasses and retain their ability to bind antigen. Using trinitrotoluene (TNT) as proof-of-concept, both competitive and displacement immunoassays were successfully conducted using sol-gel immobilized anti-TNT.[9] In the competitive immunoassays, unlabeled TNT and fluorescein-labeled TNT competed for antibody binding. Results were obtained by optically monitoring the fluorescence signal. The bioactive silica glasses with encapsulated anti-TNT were prepared as monoliths of ~1 mm thickness. As shown in Figure 3, calibration curves with the expected behavior were obtained for competitive immunoassays, and TNT concentrations on the order of one ppm were detected. Moreover, the encapsulated antibody retained the ability to differentiate between TNT and trinitrobenzene (TNB), an analog.

In addition to monoliths, we have also prepared bioactive thin films with immobilized antibodies. Although it is possible to use monoliths, for biosensing applications, the use of thin films is preferred. The primary reason is that thin films have a reduced diffusion length for the target analyte, and therefore, there is a greatly reduced response time, since diffusion

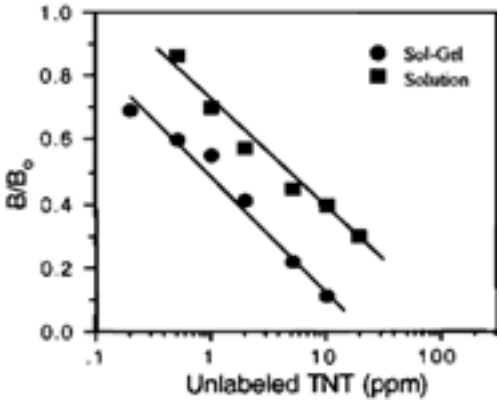

Fig. 3 Logarithmic plots of signal (B/B_o) vs. unlabeled TNT concentration for competitive immunoassays. Both sol-gel immobilized anti-TNT antibodies and surface immobilized antibodies show the expected linear behavior.
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distance varies with $t^{1/2}$. Whereas the required incubation time for 1 mm thickness monoliths was ~3–4 hours, for thin film (~1 μm thickness), the incubation time can be reduced to 20 minutes.

Anti-cortisol antibodies were immobilized in sol-gel silica thin films and used as sensing elements in an immunoassay for cortisol.[12] The antibody-doped thin films were of excellent quality and optical transparency, with a thickness of ~1 μm for films in the ''wet'' state. In our experiments, antibody-antigen binding was detected optically using fluorescent Oregon Green-labeled cortisol. As seen in figure 4, with a constant Ab concentration in the thin films, the fluorescence signal increased with increasing concentration of labeled antigen. Moreover, by raising the antibody concentration in the thin films, one can obtain higher optical signals. We conducted competitive immunoassays for cortisol using anti-cortisol doped silica thin films.

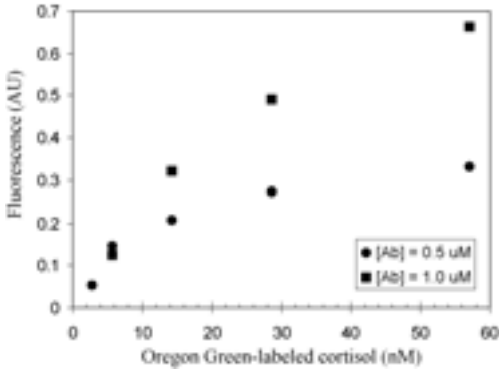

Fig. 4 Fluorescence signal as a function of OG-cortisol concentration for sol-gel silica thin films containing 0.5 μM or 1.0 μM anti-cortisol antibody. The sol-gel encapsulated antibodies retained their ability to bind antigen, and by increasing the antibody concentration, higher signals can be obtained.

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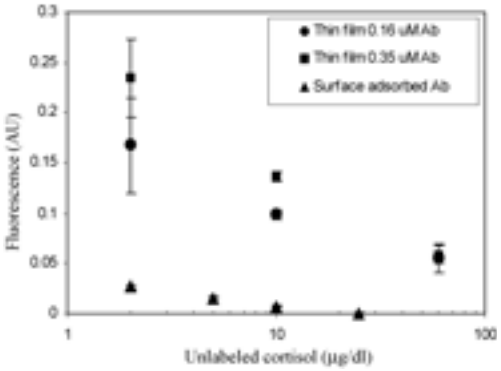

Fig. 5 Calibration curves from competitive immunoassays conducted with sol-gel silica thin films containing anti-cortisol and with surface adsorbed anti-cortisol. With sol-gel encapsulation, substantially higher signals were obtained because there was a substantially higher number of biomolecules per unit area in the sol-gel glass as compared to traditional monolayer surface adsorption.

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In competitive immunoassays with thin films, as was the case with the monoliths, the calibration curves exhibited the expected behavior, i.e. linear with a negative slope (Figure 5).

When immobilizing biomolecules, one distinct advantage of sol-gel encapsulation is the ability to create three-dimensional architectures. With traditional immobilization methods, such as surface adsorption or covalent attachment, monolayer coverage of the biomolecule is usually obtained. With sol-gel encapsulation, however, one can achieve a substantially higher number of biomolecules per unit area because of the three-dimensional nature of the bioactive glass. When comparing competitive immunoassay results for anti-cortisol encapsulated in sol-gel thin films (~1 μm thickness) and surface adsorbed on polystyrene, the fluorescence signals from the sol-gel films were as much as ten times higher than those measured using surface-adsorbed antibody (Figure 5).

4. Summary

Immobilization of biomolecules in sol-gel derived glasses leads to a wide variety of bioactive materials. The flexible processing inherent in sol-gel synthesis and the ability to monitor biological and/or chemical changes make these materials viable for biosensing. With current technology, relatively large biomolecules (e.g. proteins) are immobilized in the pores of the matrix while small analytes can diffuse through the porous network to be detected. The enhanced thermal, chemical, and storage stability observed for a number of proteins immobilized via the sol-gel method makes these materials especially attractive as sensing elements. Future work directed at continuous monitoring, along with incorporating the sol-gel sensing elements into actual devices, will enable these

materials to make a wide impact on biosensing technology.

Acknowledgements

Support for the research from NSF (DMR 0103952 and DMR 0099862) and NASA (NAG9–1252) is gratefully acknowledged. This work was also partially supported by the Center for Cell Mimetic Space Exploration (CMISE), a NASA University Research, Engineering and Technology Institute (URETI), under award number # NCC 2–1364.

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